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A Quantitative Assessment of Spermatozoan Morphology in *Nutricola confusa* and *Nutricola tantilla* (Bivalvia: Veneridae)

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Abstract. The brooding bivalves, *Nutricola tantilla* and *N. confusa* overlap in their geographic distributions, habitats, and modes and timing of reproduction. Based on previous studies we infer that males of both species release spermatozoa into the water column; while females retain developing embryos in a brood chamber. Females release fully formed juveniles and there is no pelagic larval stage. We hypothesized that the muco-ciliary processes of particle selection and retention may act on differences in sperm morphology and contribute to reproductive isolation. We extracted sperm cells from both species and quantified nine linear measurements: the lengths of the acrosome, nuclear, midpiece and tail regions, and five different width measurements. We found significant differences in the lengths of the acrosome, midpiece, and tail. We also found that *N. confusa* produces dimorphic sperm and this is the first report of sperm dimorphism in the Veneroidea. Despite the significant differences in lengths, it is likely that other prezygotic mechanisms are responsible for reproductive isolation.

INTRODUCTION

Nutricola tantilla and *N. confusa* are morphologically similar, small bivalves (<10 mm shell length), that inhabit the top 2.5 cm of soft substrata in the intertidal to shallow-subtidal zones of protected bays of western North America (Coan et al., 2000). The reported geographic range of *Nutricola tantilla* is from Prince William Sound, Alaska to Isla Cedros, Baja California and *N. confusa* occurs from Coos Bay, Oregon to Carmel Bay, California. Where their ranges overlap they are sympatric and both species are very common in Bodega Bay, California (Grosholz & Ruiz, 1995). In earlier studies, the two species have been referred to as *Transennella* and there is some disagreement over the taxonomy (Lindberg, 1990).

Females of both species are generally larger than males (Hansen, 1953; Asson-Bartres, 1988; Russell and Huelsenbeck, 1989). Hansen (1953) performed histological examinations of 371 specimens and found "5 were in a state of reversal from male to female" (p. 319). Some studies have cited this work as evidence for protandry (Kabat, 1985, 1986; Asson-Bartres, 1988) whereas Mottet (1988) attributes the size disparity to differential growth rates.

Both species lack pelagic larval stages and females brood their developing embryos and early juvenile stages in a pouch located between the inner demibranch and visceral mass (see figures 2 and 3 in Kabat, 1985 for detailed SEMs). Broods can be found throughout the year but there is seasonal variation in reproduction with higher levels during the summer and fall (Asson-Batres, 1988; Russell & Huelsenbeck, 1989). During peak periods of reproduction brood number can be as high as 300 and is a function of female size (Kabat, 1985; Russell & Huelsenbeck, 1989).

Sperm storage has not been reported in *Nutricola* (Mottet, 1988), we have not observed it, and therefore conclude that these species outcross. We infer that males release sperm into the water column because individuals produce only eggs or sperm at any one time and females retain their eggs for brooding. Sperm probably enter the mantle cavity of a female through the siphons. This fertilization mechanism, called "spermcast mating" (Bishop & Pemberton, 2006), has been proposed for other outcrossing brooding bivalves (Oldfield, 1964; Sellmer, 1967) and shown to be the method of sperm transfer for the brooding bivalve, *Mysella tumida* (O'Foighil, 1985b). Once inside the mantle cavity, the mechanics of directing sperm dorsally to the ovaries and unfertilized eggs is unknown but could involve chemotaxis and/or selective muco-ciliary activity of the gills.

Nutricola tantilla and *N. confusa* are sympatric, reproduce at the same time of year, and presumably females are exposed to sperm released from males of both species. One question that arises from these circumstances is how is reproductive isolation maintained? The purpose of our study was to quantify the gross morphology of spermatozoa from *N. tantilla* and *N. confusa* to determine whether differences in size/shape could contribute to the maintenance of reproductive isolation. This study represents the first attempt

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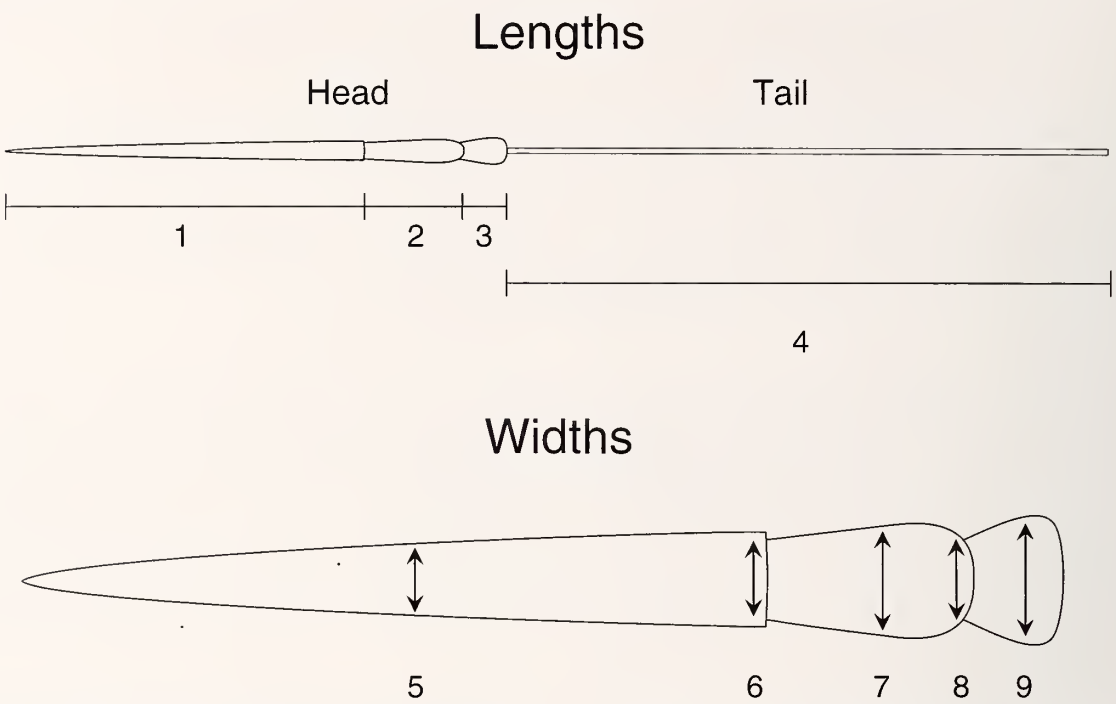

Figure 1. Schematic diagram of spermatozoa found in *Nutricola* illustrating the nine linear dimensions measured. Lengths: 1 = acrosome, 2 = nuclear region, 3 = midpiece, 4 = tail. Widths: 5 = acrosome midpoint, 6 = acrosome juncture with nuclear region, 7 = nuclear region midpoint, 8 = nuclear region juncture with midpiece, 9 = midpiece.

to quantify *Nutricola* spermatozoa morphology using electron microscopy.

METHODS AND MATERIALS

Samples of both species of clams were collected from Bodega Bay California on March 26, 2007, shipped overnight to Villanova University, separately maintained in a sea table (10°C and 32‰), and fed a mixture of phytoplankton cultures of *Tetraselmis sp.*, *Thalassiosira sp.*, *Isochrysis galbana*, and *Chaetoceros muelleri* until processed.

Attempts to induce spawning with thermal shocking methods (Castagna & Kraeuter, 1981; Deming & Russell, 1999) were unsuccessful so we resorted to extracting sperm via gonad squashes. Individuals of each species were dissected in separate containers of sea water. The gonads were removed, gently macerated, and released sperm in the sea water. Samples of the seawater were examined with a compound microscope for the presence/activity of spermatozoa. When active spermatozoa were identified, additional seawater samples with sperm were pipetted on to ploy-L-lysine coated cover slips. The sperm were fixed at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, adjusted to pH 7.4 and 900 mOsm with 0.4 M NaCl and 2 mM $CaCl_2$. After a rinse in the same buffer, samples were postfixed in 1% osmium tetroxide, in the same buffer, and rinsed again. Samples were dehydrated in an ascending series of ethanol dilutions (25%, 50%, 75%, 95%, and 100%) and critical point dried using CO_2 as transitional fluid. Cover slips were sputter coated with gold/palladium and observed in a Hitachi S-570 SEM at 5 kV.

Digital images of intact spermatozoa were captured and then measured using ImageJ software version 1.37 V (Abramoff et al., 2004). Nine separate linear measurements were recorded on each intact spermatozoan (Figure 1): lengths of the acrosome, nuclear region, midpiece and tail; and widths of each of the three head regions (acrosome, nuclear, and midpiece) as well as the widths of the boundaries between adjacent head regions.

All data were tested for normality using a Shapiro-Wilk W test and the nine linear dimensions between species were compared using a t-test when both data sets were normally distributed, or a Wilcoxon Rank Sum test when either (or both) data sets were not normally distributed. A Principle Component Analysis (PCA) was used to combine all morphometric data to visualize the degree of separation between the two species based on spermatozoa morphology. Finally, a discriminant analysis was performed to assess how many of the samples would be correctly assigned to each species based on the nine linear measurements. All

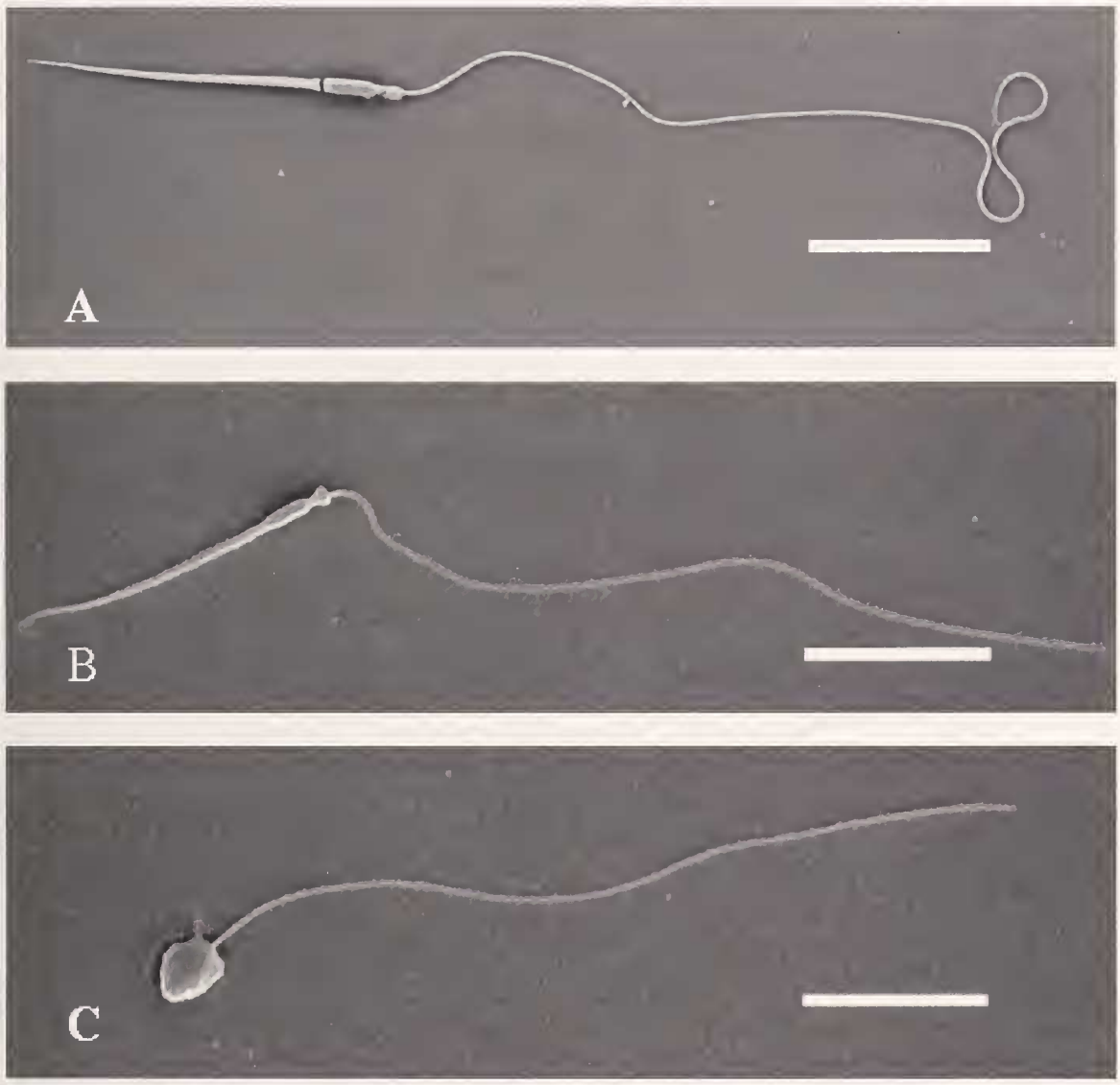

Figure 2. Examples of spermatozoa found in *Nutricola*. The scale bars = 10 μm. A. *Nutricola tantilla*. B. *Nutricola confusa*. C. This type of sperm representing a distinct morphology (rounded-head) was found only in *N. confusa*.

analyses were performed using JMP (Version 4.04, SAS Institute Inc.).

RESULTS

Active sperm were found in all samples of male clams; 3 males were found for *N. tantilla* and 2 males for *N. confusa*. Electron microscopy preparations were processed for each male and intact spermatozoa were identified and measured for both *N. tantilla* (n = 13) and *N. confusa* (n = 18). Both species exhibited markedly elongated heads and examples of these cells illustrating the three distinct regions of the head are shown in Figure 2. Furthermore, a morphologically different sperm with a round head was found only in samples from *N. confusa* (Figure 2C).

Significant differences in the lengths between the species were found in the acrosome ($Z = 2.62$, $P = .0087$), tail ($Z = 3.70$, $P = .0002$), and midpiece ($t = 4.39$, $P = .0001$) regions. In all three cases the spermatozoa of *N. tantilla* were significantly longer than *N. confusa* (Figure 3). No significant difference was found in any of the width measurements or the length of the nuclear region. The range-frame box and whiskers plots show that although the spermatozoa from *N. tantilla* are longer, there is considerable overlap with *N. confusa* (Figure 3).

The results of a PCA are displayed in Figure 4 and a

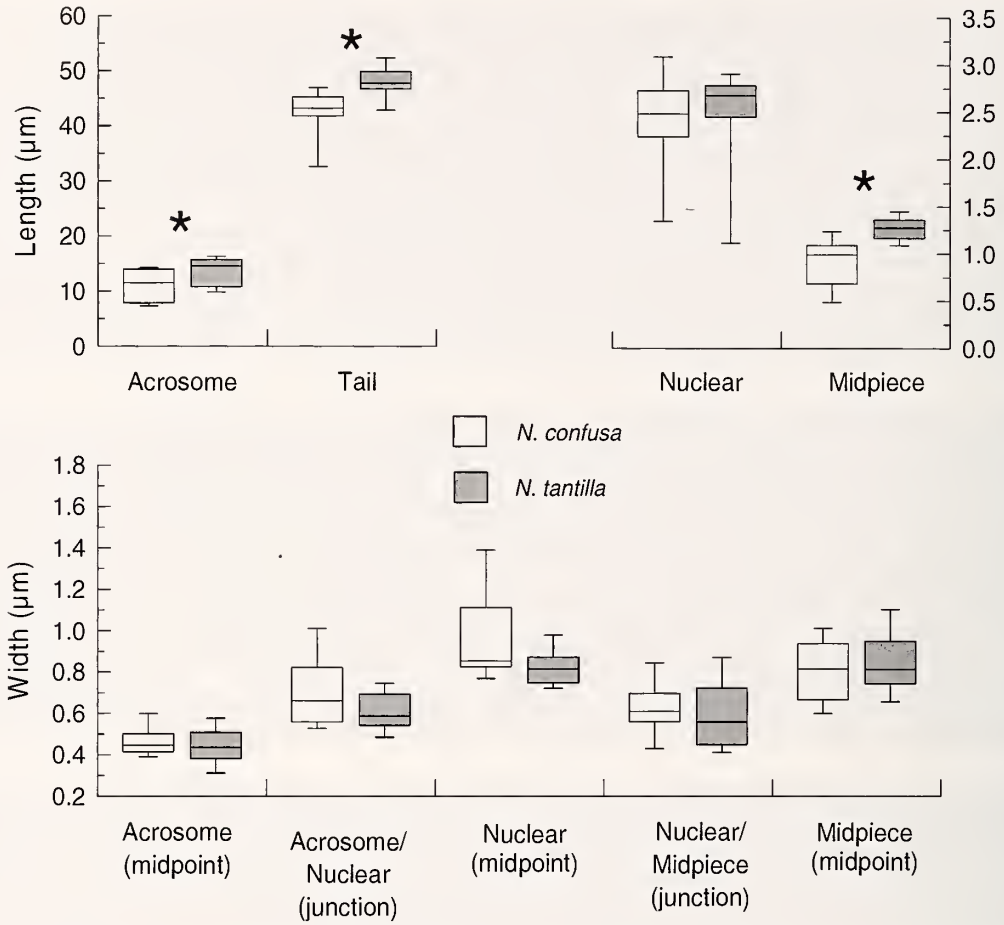

Figure 3. Box and whiskers plots of the nine linear measurements of *N. tantilla* (shaded) and *N. confusa* (open) spermatozoa. The horizontal lines within each box are the medians, the edges of the boxes are the 25th and 75th percentiles and the whiskers are the 5th and 95th percentiles. Significant differences (*) were found in the acrosome, tail, and midpiece lengths.

discriminant analysis correctly identified 87% (27 out of 31) of the measured spermatozoa.

DISCUSSION

Both species showed spermatozoa with elongated heads composed of three distinct regions (Figure 2). The designations of acrosome, nuclear, and midpiece (Figure 1) are based on the relative positions of these regions in the sperm of other taxa (Franzén, 1956) and comparison with the illustration and description of the spermatozoa of *Transennella* (= *Nutricola*) *tantilla* in (Thompson, 1973). The mean lengths of the acrosome, nuclear, and midpiece regions of *N. tantilla* from our samples are 13.68, 2.47, and 1.27 (µm) respectively, which are comparable to the lengths Thompson (1973) reported: 15.0, 2.4, and 1.0 (µm). Franzén (1983) noted that the acrosome is a "prominent structure" in bivalve spermatozoa (as is the case here) and commented on Thompson's (1973) description of *N. tantilla* spermatozoa that "in spite of its unusual proportions [it] seems to belong to the primitive type."

Spermatozoa with a distinctly different morphology were found only in *N. confusa* (Figure 2C). We did not observe any intermediate stages between the "round headed" sperm and the mature sperm with the elongated acrosomes (Figure 2B). This observation strongly suggests the presence of sperm dimorphism in *N. confusa*. Sperm dimorphism is relatively uncommon in bivalves having been reported in only a few species (Ockelmann, 1965; Jespersen et al., 2001; Jespersen & Lützen, 2001; Lützen et al., 2001; Jespersen et al., 2002; Jespersen et al., 2004; Lützen et al., 2004). We found this second type of sperm in all of the SEM preparations of *N. confusa* and in none of the preparations from *N. tantilla*. This finding is the first reported case of sperm dimorphism in the Veneroidea. Other reports of sperm dimorphism occur in the Galeommatoidea where one species in one genus can exhibit sperm dimorphism, e.g., *Kurtiella bidentata (as*

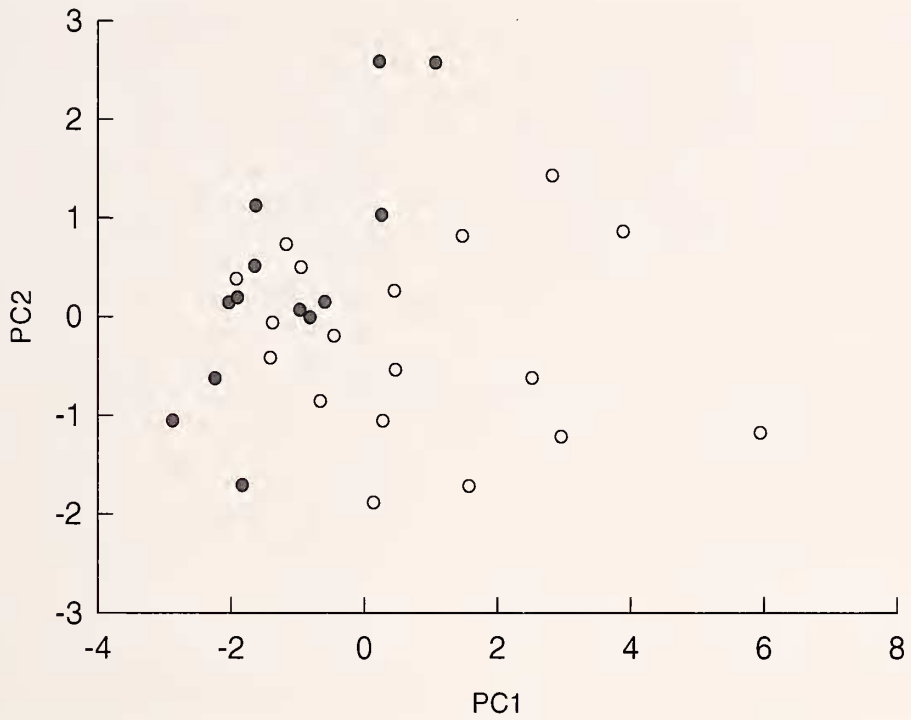

Figure 4. Results of Principle Component (PC) analysis using the nine measures illustrated in Figure 1. *Nutricola tantilla* (shaded, n = 13) and *N. confusa* (open; n = 18).

Mysella), and another closely related species does not, e.g., *M. tumida* (Ockelmann & Muus, 1978; O'Foighil, 1985a). It appears that this is the situation with the congeners *N. tantilla* and *N. confusa*.

The acrosome, midpiece and tail of the *Nutricola tantilla* sperm are significantly longer than those of *N. confusa* but no differences were found in the length of the nuclear region or any of the five width measurements (Figure 3). There is a significant difference in the overall mean lengths of spermatozoa – *N. tantilla* 65.26 µm ± 4.64 and *N. confusa* 56.93 µm ± 5.73 (± SD, Z = 3.78, *P* = .0002). Although there are significant morphological differences between the sperm in these species there is also considerable overlap in the variables measured. This point is illustrated by the PCA plot (Figure 4) which shows a limited degree of separation between the two species.

There are at least three hypotheses for the functional significance of elongated sperm heads in bivalves. Recently, Jespersen & Lützen (2007) proposed that this morphology allows sperm cells to better circumvent retention by the gills thus facilitating fertilization. Franzén (1983) found that elongated sperm heads are correlated with larger eggs and may aid in sperm penetration. Finally, Jespersen et al. (2001) proposed that the elongated sperm heads of the euspermatozoa of *Pseudopythina macrophthalmensis* may promote storage of sperm in seminal receptacles. Neither *N. tantilla* nor *N. confusa* have seminal receptacles and do not store sperm so the later hypothesis does not apply to these species. However, the unusually long sperm heads of *N. tantilla* and *N. confusa* could function in either gill circumvention or penetration of the large lecithotrophic eggs.

The study of the muco-ciliary processes of particle selection and retention in bivalves has a long and rich history (see Ward & Shumway, 2004 for review). The focus of these studies has been on feeding biomechanics and the ecological role bivalves play in benthic-pelagic coupling processes. During preingestive processing, "there are opportunities for particle selection based upon quantitative and qualitative aspects of the particles" (Ward & Shumway, 2004:85). Spermatozoa cells of spermcast-mating, brooding bivalves like *Nutricola*, are within the size-range of particles selected via the muco-ciliary processes (Mohlenberg & Riisgård, 1978) and are likely subject to these processes. The differences in sperm morphology demonstrated here while significant, are probably insufficient by themselves to account for species-specific spermatozoa recognition. These species produce hundreds of eggs compared to the hundreds of thousands produced by broadcast spawning taxa and cannot afford postzygotic isolation. Therefore it is likely that factors other than spermatozoa morphology play a role in maintaining reproductive isolation via prezygotic mechanisms.

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